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The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*

David E. Briles [a,b,*], Susan K. Hollingshead [a], Gary S. Nabors [c], James C. Paton [d], Alexis Brooks-Walter [a]

[a] *Department of Microbiology, University of Alabama at Birmingham (UAB), Birmingham, AL, USA*
[b] *Department of Pediatrics, University of Alabama at Birmingham (UAB), Birmingham, AL, USA*
[c] *Aventis Pasteur, Swiftwater, PA, USA*
[d] *Molecular Biology Unit, Woman's and Children's Hospital, North Adelaide, SA, Australia*

Abstract

Potential vaccine strategies against otitis media are to prevent (1) symptomatic infections in the middle ear and/or (2) carriage of pneumococci and thereby subsequent middle ear infections. The possibility of using immunity to virulence proteins of pneumococci to elicit immunity against pneumococci has been examined. PspA has been found to have efficacy against otitis media in animals. Vaccination with a mixture of PsaA and PspA has been observed to offer better protection against nasal carriage in mice, than vaccination with either protein alone. PspA and pneumolysin have been shown to elicit protection against invasive infections. The inclusion of a few of these proteins into the polysaccharide-protein conjugate vaccines may be able to enhance their efficacy against otitis media and might be able to constitute a successful all-protein pneumococcal vaccine. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: PspA; PsaA; *Streptococcus pneumoniae*

1. Introduction

The reservoir of *Streptococcus pneumoniae* in the human population is thought to reside largely in the asymptomatic carriage of pneumococci in the nasopharynx. Under predisposing conditions, especially following infection with respiratory viruses and blockage of the estuation tube, the pneumococci colonizing the upper airways are able to cause symptomatic otitis media (Figure 1). Vaccines could protect against otitis media by protecting against infection in the middle ear, by preventing nasopharyngeal colonization of the immunized individual, or by reduction of carriage of the population from whom at risk individuals can acquire pneumococci. For the pneumococcus the reduction of carriage would also be required for herd immunity, since it is from carriers that most pneumococci are thought to be acquired. The most successful vaccines against otitis media will probably be those that elicit herd immunity, as well as immunity to carriage and otitis. Since pneumococci also cause meningitis, bacteremia, and respiratory infections in children, it would also be desirable for a vaccine against otitis media to protect against pneumonia and invasive disease.

Otitis media is the most common infection caused by *Streptococcus pneumonia* in children in the developed world [1]. Another major cause of this otitis media, type b *Haemophilus influenzae*, has recently been brought under control in the developed world by a polysaccharide-protein conjugate vaccine [2]. This vaccine was prepared by covalently linking the type b polysaccharide to one of two different protein antigens that are highly immunogenic in children. The success of the type b conjugate vaccine has been dependent in large part on the fact that immunization with it has greatly decreased carriage of *H. influenzae*, with a resultant increase in herd immunity [2]. Since widespread immunization with the type b conjugate vaccine has caused type b *H. influenzae* to be carried at a lower frequency, the expo-

* Corresponding author. Present address: BBRB 658, 1530 3rd Ave. South, Birmingham, AL 35294-2170, USA. Tel.: +1-205-9346595; fax: +1-205-9340605.
E-mail address: dbriles@uab.edu (D.E. Briles).

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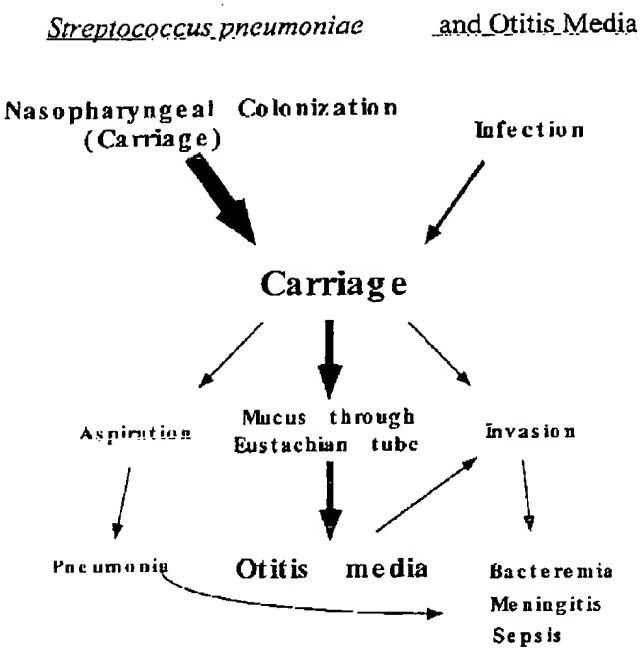

Fig. 1. Natural history of otitis media infections and other infectious caused by *Streptococcus pneumoniae*.

sure of both immunized and non-immunized children to the pathogen was decreased.

The tremendous success of the *H. influenzae* vaccine has encouraged the development of vaccines that might be able to prevent otitis media caused by *S. pneumoniae* [3]. Although antibodies to pneumococcal capsular polysaccharides can be highly protective, the polysaccharides are not immunogenic in children unless they are injected as conjugates covalently linked with immunogenic proteins such as diphtheria or tetanus toxoid. In the case of the pneumococcus, however, the problem may require a somewhat different solution. To elicit protection against type b *H. influenzae*, immunity to only a single polysaccharide is required. There are, however, at least 90 different capsular types of pneumococci [4]. For pneumococci, vaccines containing conjugates with up to 11 different polysaccharides are being developed for use in children. Even if they are as successful against otitis media as is hoped, they will not protect against strains of all capsular types. Moreover it has been observed that vaccination with the PS-protein conjugate vaccines is able to select for increased frequency of carriage with pneumococci bearing capsular types not included in the vaccine [5,6].

2. Pneumococcal proteins versus PS-protein conjugate vaccines

The pneumococcal polysaccharide-protein conjugates that have been tested in children have yielded measurable protection from otitis. However, the protection obtained was not as great as that achieved against *H. influenzae* by immunizing with the *H. influenzae* type b polysaccharide-protein conjugate. The fact that non-vaccine capsular types increased in incidence following immunization with PS-conjugate vaccines further raises the possibility that the PS-protein conjugates by themselves may not be an ideal vaccine for the prevention of otitis media. Moreover, it will probably be many years before the highly complex multi-valent capsular-protein conjugate vaccine will be inexpensive enough for use in children in the developing world who are those at highest risk of serious pneumococcal infection.

A number of laboratories around the world have been investigating pneumococcal proteins for their ability to elicit immunity to pneumococcal bacteremia, pneumonia, and otitis media [7]. These pneumococcal proteins offer several potential advantages. Since they are each virulence factors, vaccines containing mixtures of them are able to target more than one mechanism important for pneumococcal carriage and invasion. As proteins the antigens would be expected to be immunogenic in young children without the necessity to prepare chemical conjugates with other carriers. The resultant vaccine should also be less expensive since the cost associated with conjugation, purification of products, and quality control would be minimized.

Finally there is a theoretical problem with the fact that young children do not make antibodies to polysaccharides in spite of the fact that such antibodies would be enormously beneficial against otherwise fatal infections with encapsulated bacteria including pneumococci, *H. influenzae*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, and others. The selective pressure favoring childhood immune responses must have been large during the millions of years of human evolution. It is difficult to know why evolution has left this gap in the immune potential of young children, unless it was because immune responses by the very young to at least some polysaccharides were deleterious [8–11]. The use of protein, rather than polysaccharide-containing vaccines in children should avoid this potential problem.

If, however, protein-PS vaccines are found to be a safe means of eliciting protection against pneumococci, it is likely that the most efficacious vaccines would be those where the pneumococcal proteins are used as carriers, rather than the non-pneumococcal carriers presently in use. The reasons for this are three-fold. (1) By using pneumococcal proteins as carriers there will be less immunologic conflict with other vaccines that also contain diphtheria or tetanus toxoid. (2) Any immunity to the carrier proteins would complement that to the polysaccharides and could result in superior and broader protection. (3) Anamnestic responses to any subsequent pneumococcal infections should be improved. T-dependent B cell responses are best driven by antigens that can stimulate cognate T-cell help. Such

antigens are those where T and B cell epitopes are present on the same macromolecular structure. When antigens containing both T and B cell epitopes are used for immunization antigenic-specific B-cells are able to present to T-cells cytoplasmically processed T-cell epitopes in the context of class II MHC on the B-cell surface. If PS-protein conjugate vaccines utilize pneumococcal proteins as carriers the anamnestic responses stimulated by infection with pneumococci could benefit from both the memory T and B cell populations stimulated by the original immunization with the vaccine. On the other hand, if non-pneumococcal proteins such as diphtheria toxoid or tetanus toxoid are used as carriers, there may be little T cell help for the PS antigens presented by the pneumococcus.

Finally, a vaccine containing protection-eliciting pneumococcal proteins in addition to capsular polysaccharides should provide less opportunity for selection for strains with capsular types not included in the vaccine, or for that matter strains that might eventually figure out how to evade immunity to the protection-eliciting proteins.

3. Pneumococcal proteins that are vaccine candidates

There have been several extensive recent reviews on the potential use of proteins and other non-capsular antigens as pneumococcal vaccines [7,11–13]. This review will focus on results relating to otitis media, and vaccines with mixtures of different pneumococcal proteins.

The proteins PspA, pneumolysin, PsaA, and PspC have been shown to be efficacious against pneumococci in animal models [7,11–14]. Other proteins like neuraminidase, and hyaluronidase have been studied less, but have also yielded encouraging results, and may find value as components of protein vaccines [7,15–17]. IgA1 protease might be expected to have an important role in protection against mucosal immunity during carriage or possibly otitis media [18]. Since this enzyme does not cleave mouse IgA, tests of its efficacy in animals have not been carried out.

Table 1
Effect of anti-PspA on otitis media in rats[a]

Immunogen	Numbers of rats	% Otitis
	Otitis:No otitis	
PspA+CFA	0:8[b]	0
Saline only	3:1	75
CFA only	2:2	50

[a] This data is adapted from White et al., 1999 [19].
[b] PspA vs. pooled controls; $P = 0.026$ by Fisher's exact test.

Table 2
Protection against nasal carriage in mice by intranasal immunization with different antigens[a]

Preparations eliciting protection against carriage	References
Killed pneumococci	[21]
Autolysed pneumococci	[21]
Homologous native PspA	[22]
6B PS-protein conjugate	[22]
rPsaA + heterologous rPspA	[17]

[a] All immunogens were given with cholera toxin B subunit as adjuvant. In each case protection was statistically significant compared to mice immunized with adjuvant alone.

4. Otitis media

The only protein that has provided evidence of protection against otitis media per se has been PspA. This protein has been shown to be able to elicit protection against otitis media in a rat model (Table 1), where the course of the disease was monitored by observation of the tympanic membrane of the infected rats [19]. These findings with rats appear to be supported by recent preliminary studies of Dr Steven Pelton (personal communication) which indicate that PspA is protective against otitis media in chinchillas.

A protein-containing pneumococcal vaccine could also provide protection against otitis media by eliciting mucosal immunity against pneumococcal carriage, which is believed to precede clinical otitis media [20]. Any vaccine that could eliminate or greatly reduce carriage rates in a high enough frequency of children could break the cycle of carriage and might be able to largely eliminate the organism from the target population.

5. Nasal carriage

Using a mouse carriage model we have been able to clearly shown that intranasal immunization can largely eliminate nasal carriage of *S. pneumoniae* (Table 2). Immunization with heat-killed pneumococci, autolysed pneumococci, or homologous native full-length PspA virtually eliminated nasal carriage [21,22]. Immunization with a capsular type 6B-tetanus toxoid conjugate also reduced carriage. For reasons we can only speculate on, immunization with truncated, recombinant, heterologous PspA has not been as protective against carriage [17] as was homologous full length native PspA [22]. In contrast, immunization with recombinant lipidated PsaA has been able to largely eliminate carriage with pneumococci [17].

Immunization with a combination of PsaA and PspA, however, was found to be much more protective against carriage with capsule type 6B strain L82016 than immunization with either of the proteins alone (Fig. 1). Using a capsular type 23 strain, some protection against carriage was observed after immunization with PsaA but not

Table 3
Geometric mean levels of antibody to pneumococcal proteins after intranasal immunization

Antigen	Immunization (μg)[a]	μg Antibody/ml	
		Saliva	Serum
PsaA + CTB	0.5	1.0	257
PspA + CTB	0.5	0.014	31
PdB + CTB	10.0	0.0019	2.15

[a] Mice were given three i.n. immunizations per week for 3 weeks and bled 3 weeks after the last immunization. CTB (0.4 μg) was given with the antigens during the first 2 weeks only. Data abstracted from reference [17].

PspA. As for strain L82016, however, we observed that immunization with PsaA together with PspA yielded better protection against carriage than PsaA alone ($P < 0.05$) [17](Fig. 2A).

In the studies depicted in Fig. 2A and B the mice were immunized intranasally. The PsaA that was used for immunization was lipidated and was immunogenic intranasally without adjuvant. The rPspA used was not lipidated and required an adjuvant for intranasal (i.n.) immunization. Thus, for these studies the mixtures of antigens and the antigens by themselves were always administered together with added cholera toxin B subunit (CTB) [17]. It was observed that PsaA elicited almost 100 times as much mucosal antibody as did PspA (Table 3). This is probably a reflection of the

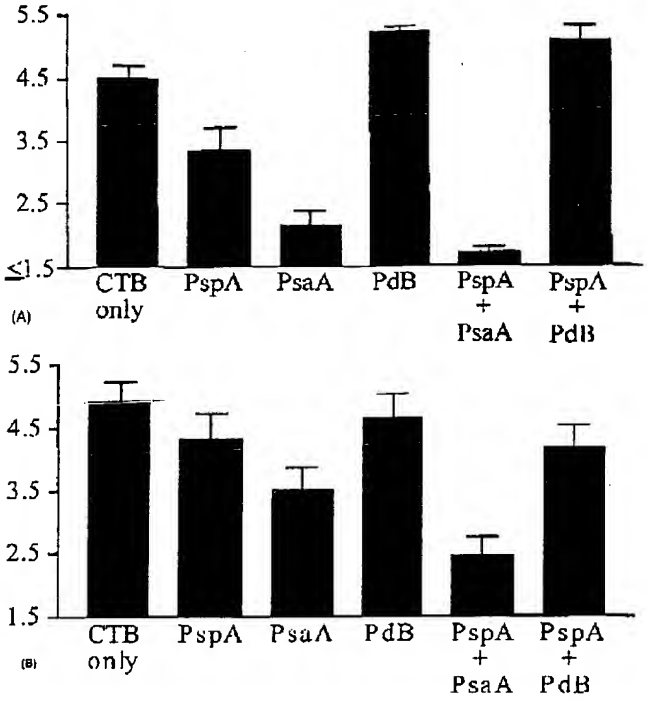

Fig. 2. Protection against nasal carriage with capsular type 6b strain L82016(2A) and capsular type 23 strain E134(2B). Mice were immunized intranasally with 0.5μg of rPspA, 0.5μg of rPsaA, and/or 10μg of rPdB (a detoxified mutant protein of pneumolysin). Cholera toxin B subunit (CTB) was used as an adjuvant. Mice were challenged with about one million CFU of each challenge strain three weeks post immunization. The number of CFU per nose was determined one week post challenge as described previously [22]. This data has been published previously [17].

Table 4
Ability of full-length native PspAs to elicit immunity to pneumococci expressing PspAs of different clades and families (results expressed as median days alive)

Challenge strain[a]				Vaccine PspA[b]			
Name	Capsular type	PspA clade	PspA family	D39	WU2	BG9739	None
D39	2	2	1	4.5*[c]	>21*		2
WU2	3	2	1	>21*	>21*	>21*	2
A66	3	1+2[d]	1	>21*	>21*	>21*	2
EF10197	3	2	1	>21*	>21*	>21*	2
ATCC6303	3	5	2	>21*			5
BG9739	3	1	1	3*	>21*	6*	2
EF3296	4	3	2	5*	5*	5*	2
EF5668	4	4	2	6*	2	>21*	3
L81905	4	1	1	5*	5*	8*	2
DBL5	5	2	1	4*		3	2
EF6796	6A	2	1	>21*			1
DBL6A	6A	1	1	>21*	9*	13*	6
BG9163	6B	2	1	>21*		>21*	9
BG7322	6B	2	1	>21*	>21*	15*	7

[a] Mice were challenged intravenously with 30–100 times the 50% lethal dose of each strain.

[b] Mice were immunized subcutaneously with PspA isolated from pneumococci using a choline-sepharose column. Immunizations were carried out with Freund's complete adjuvant. Data in the 'None' column comes from mice immunized with complete Freund's adjuvant lacking PspA 30. In cells where data is presented, there was no experiment.

[c] Data are expressed as median days alive. * Indicates significantly different from 'none' at $P < 0.05$ by Wilcoxon's two-sample rank test. An empty data cell indicates that no experiment was conducted. This table was published previously prior to determination of PspA clades and families of the relevant strains [30].

[d] Strain A66 produces two PspAs, one of PspA clade 1 and one of clade 2. Both are PspA family [1].

Table 5
Cross-protection resulting from immunity to recombinant PspAs of different PspA clades and families (based on published studies [32] for which we now know the clades and families of the PspA immunogens (Hollingshead et al. in manuscript submitted))

Immunogen[a]		Challenge strain			Days alive[b]			Alive:dead[c]			Reference
Donor	PspA Clade/family	Name	PspA Clade/family	Capsule type	Immune	Control	*P* value	Immun	Control	*P* value	
D39[d]	2/1	WU2	2/1	3	>16	1.3	<0.005	8:2	1:9	0.006	[31]
		A66	1/1 & 2/1[e]	3	>16	1.7	<0.001	11:0	0:7	0.191	[31]
		EF6796	2/1	6A	>16	5.5	<0.001	9:5	1:11	0.006	[31]
		D39	2/1	2	2.4	1.2	n.s.	0:9	0:13	n.s.	[31]
DBL5	2/1	WU2	2/1	3	>21	3	0.0038	4:0	1:5	0.048	[32]
		BG7322	2/1	6B	>21	8	0.0061	4:0	0:7	0.003	[32]
BG9739 or L81905[f]	1/1	WU2	2/1	3	>21	3	0.0037	8:2	1:5	0.035	[32]
		A66	1/1 and 2/1	3	>21	3	0.008	8:0	1:6	0.001	[32]
		DBL6A	1/1	6A	>21	4	0.0022	5:3	0:7	0.026	[32]
		BG7322	2/1	6B	13	8	0.014	2:6	0:7	n.s.	[32]
		DBL5	2/1	5	3.5	2	0.029	0:8	0:8	n.s.	[32]
		BG9739	1/1	4	5	3	0.0093	1:7	0:7	n.s.	[32]
		L81905	1/1	4	6	4	0.0426	0:8	0:6	n.s.	[32]
EF5668	4/2	WU2	2/1	3	>10	2	0.943	8:0	0: 6	0.0003	[33]
		A66	1/1 and 2/1	3	>10	2.5	0.008	7:1	0:6	0.0047	[33]
		EF5668	4/2	4	>10	2	0.002	5:0	1:4	0.048	[33]
		BG7322	2/1	6B	>10	3	n.s.	5:1	1:5	n.s.	[33]

[a] These results have been calculated from previously published data [32]. Immunizations with rPspA in complete Freund's adjuvant. Control mice received identical preparations with Freund's complete adjuvant without PspA. Mice were challenged with 10 100 lethal dose 50 U of the indicated challenge strains by the i.v. route. Survival was monitored for 21 days [32].
[b] Statistical differences calculated with the Wilcoxon's two-sample rank test.
[c] Statistical differences calculated with the Fisher's exact test. 'n.s' means not statistically significant.
[d] Data are pooled from immunization with R × 1 and D39 PspAs, which have a common origin and are thought to be identical.
[e] A66 expresses two PspAs; one is clade 1 and the other is clade 2.
[f] Data for immunizations with BG9739 and L82015 PspAs were pooled to obtain larger numbers in each treatment group. Both PspAs were clade 1 family 1.

Table 6
Median days of death of mice immunized subcutaneously with PspA after intravenous challenge with *Streptococcus pneumoniae*[a]

Challenge strain[b]	A66.1	D39	BG7322	BG9739	DBL2	DBL5
Capsular type	3	2	6B	4	2	5
Alum only	2	3	8	4	8	2
PspA	>21*[c]	7, >21*[d]	>21**	4	>21**	5, >21**[d]
PdB	2	3	8	4	6.5	2
PspA + PdB	>21**	7, >21*[d]	>21**	6	>21*	4.5*

[a] CBA/N mice were immunized with 1.0 μg rPspA and/or 20 μg rPdB (a non-toxic mutant of pneumolysin) adsorbed to alum. The rPspA used for immunization came from strain Rx1 (clade 2, family 1). rPspA used for immunization comprised the α-helical portion of PspA with a C-terminal poly-histidine tail.

[b] All challenge strains are PspA clade 2 family 1, except for BG9739 which is clade 1 family 1, and A66.1 which expresses a clade 1 PspA, as well as a clade 2 PspA. Mice were challenged intravenously with between 30 and 100 times the 50% lethal dose of pneumococci.

[c] * and ** indicate difference in time to death from the alum control group at $P<0.05$ or $P<0.01$, respectively, as determined by a Wilcoxon's two-sample rank test.

[d] 7, >21 and 5, >21 indicate that exactly half of the mice lived (>21) and exactly half of the mice died by day 7 or 5, respectively.

Table 7
Pneumococcal protein vaccine candidates[a]

Proteins	Function	Protects in animal studies against[b]			
		Carriage	Otitis media	Pneumonia	Sepsis
PspA	Anti-complement	+	+	+	++
Pneumolysin	Anti-host defense	−		+	+
PsaA	Colonization	++		−	−
PspC	Colonization	+ +			+
IgA1 protease	Colonization?	?			
PspA + PsaA		+++		+	
PspA + Pneumolysin		+		+ +	+++

[a] This table has been expanded from a similar one published in an earlier review [15].

[b] Where no result is indicated experiments have not been conducted.

advantage of lipidation for i.n. antigenicity. In any case, in view of the tremendous differences in the amount of mucosal antibody to PsaA and PspA, it is possible that antibodies to PspA might be much more protective against carriage if they had been produced in quantities comparable to those elicited by PsaA. To this end, it should be noted that a lipidated rPspA has been produced, and was found to be much more immunogenic than the non-lipidated molecule by the i.n. or subcutaneous (s.c.) route [23]. Full-length native PspA has also been found to be more immunogenic than truncated rPspA, and might also be useful for elicitation of mucosal immunity [24].

New strategies, of mucosal immunization, including the procedure used above with PspA and PsaA, are known to elicit circulating, as well as mucosal antibody [22,25,26]. PspA, unlike PsaA is strongly protective against sepsis and bacteremia. Thus, mucosal immunization with PspA should not only be able to contribute to protection against carriage, but should also produce circulating antibody able to protect against sepsis, pneumonia, and otitis media. It is possible, in fact, that mucosal priming followed by an intramuscular boost might provide optimal protection against both sepsis and carriage. It has been shown that in man intramuscular immunization with the type b *H. influenzae* polysaccharide-conjugate vaccine can elicit local mucosal antibody. It is assumed that this is because there had been previous natural mucosal priming [27]. If this is true then intramuscular or subcutaneous boosting following mucosal priming might also be able to boost the mucosal responses.

6. PspA cross-protection

PspA exhibits variation in sequence and epitopes. In spite of this variation individual PspAs are immunologically very cross-reactive. Immunization with a single PspA elicits at least some antibody cross-reactive with all other PspAs tested [28,29] and is able to protect against infections with strains of *S. pneumoniae* expressing diverse PspAs. This cross-protection has been observed with mouse immunity to full-length PspA [11,30] (Table 4), recombinant PspA [31–33](Table 5) and also by injecting human antibody to PspA into mice [15] (Fig. 3). Based on amino-acid sequence comparisons it has been possible to divide all PspAs into six different clades (Susan Hollingshead, manuscript submitted) [29]. Five of these clades include at least 98% of over 119 pneumococcal

Table 8
Pneumococcal protein vaccine candidates[a]

Proteins	Function	Protects in animal studies against[b]			
		Carriage	Otitis media	Pneumonia	Sepsis
PspA	Anti-complement	I	I	÷	++
Pneumolysin	Anti-host defense	–		+	+
PsaA	Colonization	++		–	–
PspC	Colonization	I I			+
IgA1 protease	Colonization?	?			
PspA + PsaA		+++		+	
PspA + Pneumolysin		I		++	+++

[a] This table has been expanded from a similar one published in an earlier review [15].
[b] Where no result is indicated experiments have not been conducted.

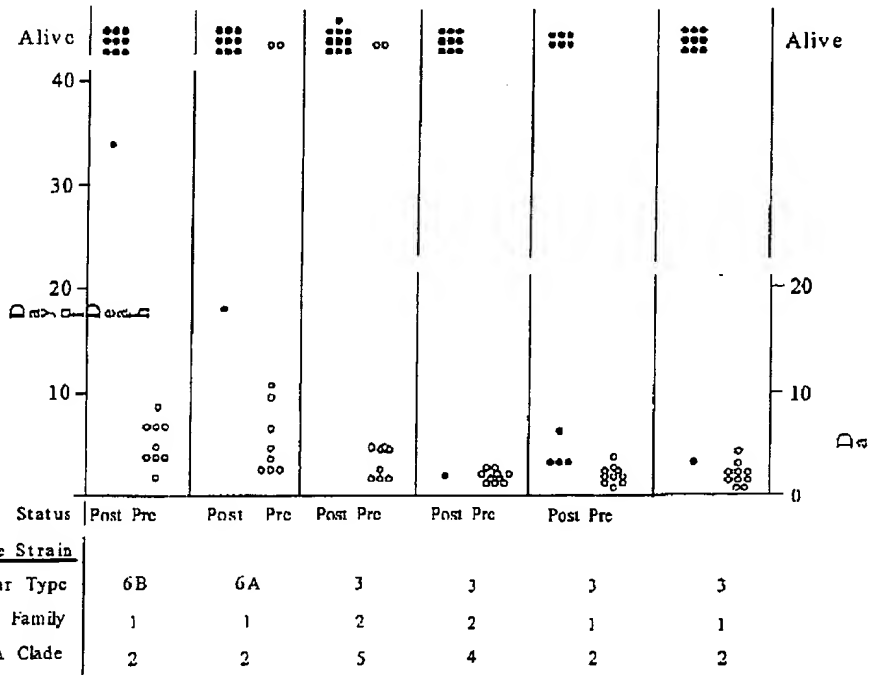

Fig. 3. Passive cross-protection against 6 strains of pneumococci with 0.1 ml of a 1/25 dilution of serum from a single volunteer immunized with recombinant clade 2 PspA from strain Rx1 adsorbed to alum. From left to right the challenge strains were BG7322, EF6796, ATCC6303, 3JP3670, EF10197 and A66.1. In each case protection was significant at $P < 0.009$ by Wilcoxon two sample rank test. This figure is adapted from (Briles et al., submitted for publication).

strains that have been examined (Susan Hollingshead et al., in preparation). These five clades have been grouped into two families based on cross-reactivity patterns detected by polyclonal antisera.

Most members of PspA families 1 and 2 can be identified (and distinguished from each other) based on the relative strength of their reactivity with rabbit antisera raised against family 1 or 2 PspAs. The individual clades within these families were seldom distinguishable with such antisera, emphasizing the immunologic similarity of PspA clades within each family (Susan Hollingshead et al, in preparation).

7. PspA and pneumolysin

In other studies it has been found that immunization of a detoxified derivative of pneumolysin may provide added protection against invasive infection with *S. pneumoniae*. For a large number of pneumococcal strains given intravenously, it is difficult to shown an enhancement of protection by adding pneumolysin to PspA (Table 6). However, some strains of certain capsular types are less affected than others by immunity to PspA (Tables 4 and 6). Using such strains it has been possible to show a strong synergy between the ability of pneu-

molysin and PspA to elicit protection against infection, particularly when the mice are challenged intraperitoneally with very high numbers of pneumococci [16] (Table 7). This synergy is also apparent when mutants are made that block the production of PspA and pneumolysin in these same strains [34]. Thus, although PspA appears to be particularly effective for eliciting protection from bacteremia and sepsis, pneumolysin may be particularly important for eliciting immunity in cavity infections. The importance of pneumolysin in cavity infections has been confirmed in recent studies where PspA and pneumolysin were both found to be partially protective against infection in a mouse pneumonia model. The combination of PspA and pneumolysin elicited better protection, however, than either protein by itself (Briles et al., manuscript in preparation).

8. Multi-protein vaccines

Although future discoveries will continue to have an impact on the formulation of protein vaccines, it is now clear that at least some of the pneumococcal proteins may be very useful. Moreover, combinations of different proteins appear likely to be able to provide the best protection against the broadest diversity of strains and disease types. Table 8 provides a summary of the relative protective capacities of several of the different pneumococcal proteins against bacteremia and sepsis, pulmonary infection, otitis media, and carriage. Based on these considerations it would appear that vaccines designed to protect against invasive infections (bacteremia, sepsis, or pneumonia) should include PspA and pneumolysin, where as vaccines designed to protect against carriage should probably contain PsaA and PspA (and/or possibly PspC). A vaccine to provide herd immunity (protection against carriage), as well as protection from invasive infection might well contain PspA, PsaA, as well as pneumolysin. If the focus of the vaccine were only on otitis media, then PspA and PsaA should both be included; PspA, since it is the only protein known to elicit any protection against middle ear infections, and PsaA, since in combination with PspA, it elicits especially strong protection against carriage.

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